PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
84 809		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/PT 00/ 00005	31/05/2000	31/05/1999
Applicant		
INSTITUTO SUPERIOR DE AGRONOMIA		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established by this Authority to read as follows:

   CULTURE MEDIUM FOR DETECTION OF DEKKERA AND BRETTANOMYCES

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. --

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/PT 00/00005

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	P. CHATONNET ET AL.: "The origin of ethylphenols in wines" JOURNAL OF THE SCIENCE OF FOOD AND AGRICULTURE., vol. 60, no. 2, 1992, pages 165-178, XP002148620 ELSEVIER APPLIED SCIENCE PUBLISHERS. BARKING., GB ISSN: 0022-5142 cited in the application page 166, column 2 -page 167, column 2 --- -/--	1-16

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 September 2000	13/10/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Griffith, G

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/PT 00/00005

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 122, no. 9, 27 February 1995 (1995-02-27) Columbus, Ohio, US; abstract no. 101322, D. A. N. EDLIN ET AL.: "The biotransformation of simple phenolic compounds by Brettanomyces anomalus" page 574; column 1; XP002148621 abstract & FEMS MICROBIOL. LETT., vol. 125, no. 2-3, 1995, pages 311-316,	1-16
A	CHEMICAL ABSTRACTS, vol. 106, no. 3, 19 January 1987 (1987-01-19) Columbus, Ohio, US; abstract no. 15531, T. HERESZTYM: "Metabolism of volatile phenolic compounds from hydroxycinnamic acids by Brettanomyces yeast" page 331; column 1; XP002148622 abstract & ARCH. MICROBIOL., vol. 146, no. 1, 1986, pages 96-98,	1-16

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PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 12 February 2001 (12.02.01)	
International application No. PCT/PT00/00005	**Applicant's or agent's file reference** 84 809
International filing date (day/month/year) 31 May 2000 (31.05.00)	**Priority date** (day/month/year) 31 May 1999 (31.05.99)
Applicant LOUREIRO, Virgílio Borges et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

15 December 2000 (15.12.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Olivia TEFY

Telephone No.: (41-22) 338.83.38

PCT/PT00/00005

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

FERREIRA MAGNO, Fernando, António
Rua Das Flores, 74 - 4º Andar
P-00-195 Lisboa
PORTUGAL

Date of mailing (day/month/year) 05 July 2000 (05.07.00)	
Applicant's or agent's file reference 84 809	**IMPORTANT NOTIFICATION**
International application No. PCT/PT00/00005	International filing date (day/month/year) 31 May 2000 (31.05.00)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 31 May 1999 (31.05.99)
Applicant INSTITUTO SUPERIOR DE AGRONOMIA et al	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.

3. An asterisk(*) appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, the attention of the applicant is directed to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

4. The letters "NR" appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, the attention of the applicant is directed to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
31 May 1999 (31.05.99)	102306	PT	20 June 2000 (20.06.00)

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer

Anman QIU

Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
84 809		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/PT00/00005	31/05/2000	31/05/1999
International Patent Classification (IPC) or national classification and IPC		
C12Q1/04		
Applicant		
INSTITUTO SUPERIOR DE AGRONOMIA et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 6 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
15/12/2000	23.08.2001
Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Thumb, W Telephone No. +49 89 2399 7350

Form PCT/IPEA/409 (cover sheet) (January 1994)

[redacted]INTERNATIONAL PRELIMINARY [redacted]EXAMINATION REPORT

International application No. PCT/PT00/00005

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1,3-5,8	as originally filed			
2,6,6a,7	as received on	06/06/2001	with letter of	04/06/2001

Claims, No.:

12 (part),13-16	as originally filed			
1-11,12 (part)	as received on	06/06/2001	with letter of	04/06/2001

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   - ☐ the description, pages:
   - ☐ the claims, Nos.:
   - ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-16
	No:	Claims	
Inventive step (IS)	Yes:	Claims	10
	No:	Claims	1-9, 11-16
Industrial applicability (IA)	Yes:	Claims	1-16
	No:	Claims	

2. Citations and explanations
   see separate sheet

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. The amendments filed with the letter dated 4.6.2001 do not appear to introduce subject-matter which extends beyond the content of the application as originally filed and therefore comply with the provisions of Article 34(2)(b) PCT.

2. Reference is made to the following document:

   D1: P. CHATONNET ET AL.: 'The origin of ethylphenols in wines' JOURNAL OF THE SCIENCE OF FOOD AND AGRICULTURE., vol. 60, no. 2, 1992, pages 165-178, cited in the application.

3. Novelty

   Claims 1-16, referring to a differential culture medium for the enumeration of yeasts of the *Dekkera* and *Brettanomyces* genus, containing, among other components, ethanol and p-coumaric acid, and the use of said medium, are novel within the meaning of Article 33(2) PCT, since said medium is not known from the state of the art known to the examining authority.

4. Inventive step

4.1 Document D1, which is considered to represent the most relevant state of the art, discloses media for culturing yeasts of the genus *Dekkera* and *Brettanomyces*.
A solid medium comprises agar (20 g/l), nutrition components, bromocresol green (0.03 g/l), cycloheximide (0.005 g/l), and 3% ethanol, the latter resulting from the addition of penicillin, gentamicin sodium sulphate and diphenyl in ethanol-solution to the medium. The pH is adjusted to 4.8 (page 167, column 1, lines 37-51).
The decarboxylation of p-coumaric acid by the yeasts is investigated in YPG liquid medium, containing 100 mg/l p-coumaric acid. The culture is continued for 3 days (page 167, column 2, lines 10-27).
The only yeasts which are capable of synthesising the 4-ethylphenol from the p-coumaric acid are *Brettanomyces/Dekkera* (page 171, column 2, lines 15-19).

4-Ethylphenol is known to possess an intense odour (page 168, column 2, lines 7-9).

4.2 The subject-matter of present claim 1 differs from the teaching of D1 in that p-coumaric acid is added to the *Dekkera/Brettanomyces*-specific medium, described in paragraph 1 of item 4.1 above.
The underlying objective problem may therefore be seen in including an additional compound in a medium suitable for detection of yeasts of the *Dekkera/ Brettanomyces* genera into a culture medium.
However, since it is already mentioned in D1 that *Dekkera* and *Brettanomyces* are the only yeasts capable of transforming p-coumaric acid into 4-ethylphenol and the characteristic odour of said compound is also well known in the art it would be obvious for the skilled person to include p-coumaric acid into a cell culture medium for specific amplification of the above-mentioned yeasts to arrive at a solution to the above stated problem.
The applicant argues that the essential technical feature of the present application is the use of ethanol as the only non-fermentable energy source in the medium. This is, however, not reflected by the broad wording of claim 1, which refers to a medium comprising (emphasis added) components including ethanol and p-coumaric acid. It is therefore not excluded that additional components may be present in the medium of claim 1 (e.g. sugars as an additional energy source). In the present wording, the only difference between the subject-matter of claim 1 and the culture medium disclosed in D1 is the inclusion of p-coumaric acid in the medium which cannot be considered as being inventive following the argumentation put forward in the preceding paragraphs.
Claim 1 therefore does not meet the requirements of Article 33(3) PCT.

4.3 Dependent claims 2-9 and 11 do not contain any features which, in combination with the features of any claim to which they refer, meet the requirements of the PCT in respect of inventive step, the reasons being as follows:
The amount of ethanol specified in claim 2 appears to be normal design option which the skilled person would choose in, accordance with circumstances, taking into consideration that the inhibitory effect of ethanol on the growth of a number of microorganisms is well known in the art.
The subject-matter of claims 3, 5, and 7 is disclosed in D1 (see item 3.1 above).

The amount of bromocresol green disclosed in claim 6 differs only marginally from the amount used in D1.
The subject-matter of claims 4 and 8 appears to be a mere selection among a number of known possibilities, not associated with a surprising technical effect, from which the skilled artisan would select without the exercise of an inventive activity (see also PCT Guidelines, IV-8.8(C1)(i)).
Claim 9 is obvious since the use of a medium with a specific composition either as a liquid or as a solid medium is trivial.
Choosing a certain order of mixing compounds of a medium and the selection of suitable sterilization steps (claim 11) is a practice well known to the person skilled in the art, and as such not suitable to confer an inventive activity.
Claims 2-9 and 11 therefore do not meet the requirements of Article 33(3) PCT.

4.4 Claims 12-16 referring to the use of said medium to detect *Dekkera/Brettanomyces* in food and beverages and for culturing said organisms is obvious for the skilled person, for example in view of the teaching of D1, referring to the detection of *Brettanomyces/Dekkera* in red wine, and wherein also the negative effects of phenolic compounds produced by said yeasts on the beverage quality are acknowledged (see page 165, abstract, lines 1-9).
Claims 12-16 can therefore also not be considered as comprising inventive subject-matter (Article 33(3) PCT).

4.5 Claim 10 differs from the teaching of document D1 in that it has a defined composition containing ethanol as the only non-fermentable energy source and p-coumaric acid.
The underlying problem may therefore be seen in providing a medium having a composition for selective growth of yeasts of the *Dekkera* and *Brettanomyces* genera.
The known state of the art does neither disclose nor render obvious that the use of a medium containing ethanol as the only non-fermentable energy source can be used to provide for differential growth of *Dekkera* and *Brettanomyces* genera compared to other yeast and bacterial species.
Hence, claim 10 meets the requirements of Article 33(3) PCT.

enumeration, as well as their later identification in food and beverages, rather difficult, this being generally accomplished through the use of very slow, work intensive, and technical skill demanding classical techniques or through molecular biology techniques, involving the use of expensive reactants, molecular probes or primers not always promptly available in the market, and of skilled operators.

It was possible to establish beyond any doubt that these yeasts are involved in the production of a serious organoleptic defect in wines - "horse sweat" - particularly in those that are aged in oak casks. (Chatonnet, *et al.* 1992, *J. Sc. Food Agric.*, 60,165-178). Since then, their detection and enumeration in wines became essential, arising the need for the development of swift methods for that effect. The field bibliography discloses suitable means for the detection and enumeration of yeasts of the *Dekkera* and *Brettanomyces* genera based on this species resistance to cycloheximide and their acidifying ability (Chatonnet, *et al.* 1992, *J. Sc. Food Agric.*, 60,165-178; Fugelsang, K. *et al.*1993. Ed. Barry H. Gump. ACS Symposium series 536, *American Chemical Society*, Washington. Cap. 7, 110-119; Alguacil, M. *et al.* 1998. *Aliment. Equipos Tecnol.* 10, 81-85). However, the disclosed media were not entirely satisfactory, since they were not selective enough to prevent the growing of fast developing species, and also were not totally differential.

Therefore, there is a real and effective need for a culture medium and method for the easy and swift identification of yeasts of the *Dekkera* and *Brettanomyces* genera, namely to provide the food and beverage industry a swift method for the isolation, differential detection and enumeration of yeasts of the *Dekkera* and *Brettanomyces* genera.

Description of the Invention

It was surprisingly found that using a culture medium containing ethanol (1) and *p*-cumaric acid for *Dekkera* and *Brettanomyces* genera yeasts growth, these

(1) as the only energy source

Further, the medium according to the invention is useful for inclusion in yeast identification galleries.

Preferred Embodiments of the Invention

~~In a preferred embodiment, the present invention refers to a culture medium for the differential detection and enumeration of yeasts of the *Dekkera* and *Brettanomyces* genera which comprises "Yeast Nitrogen Base" as a nutrient base, ethanol as a non-fermentable energy source, *p*-cumaric acid as an aromatic compound (4-ethylphenol) promoting substrate, bromocresol green as an acid-base indicator with turning points in the acid range, an antibiotic that inhibits several species of yeasts (cycloheximide), and a bacterial growth inhibitor antibiotic.~~

Insert page 6a

In this embodiment of the invention, after culture medium inoculation with a sample containing yeasts of the *Dekkera* and *Brettanomyces* genera, which may be a previously isolated sample of these yeasts, or a mixed sample of yeasts and/or yeasts and bacteria, and incubation under advantageous growth conditions for these yeasts genera, after about 5 to 12 days, identification is possible by a culture medium color change, from blue to yellow, development of cream colored colonies and the characteristic phenol-like aroma.

The present invention is further illustrated by means of the following examples, which are intended only to exemplify and by no means limit the scope of the invention.

Examples

Example 1:

Preparation of a culture medium according to the invention

The culture medium, object of the present invention, can be prepared using

In a preferred embodiment, the present invention refers to a culture medium for the differential detection and enumeration of yeasts of the *Dekkera* and *Brettanomyces* genera which comprises "Yeast Nitrogen Base" as a nutrient base in an amount from 5 to 10 g/L, preferably 6.7 g/L, ethanol as a non-fermentable energy source in an amount from 32 to 96 g/L, preferably 48 g/L, *p*-cumaric acid as an aromatic compound (4-ethylphenol) promoting substrate, in an amount from 0.05 to 1.0 g/L, preferably 0.1 g/L, bromocresol green as an acid-base indicator with turning points in the acid range, an antibiotic that inhibits several species of yeasts (cycloheximide) in an amount from 0.004 to 0.1 g/L, preferably 0,01 g/L, and a bacterial growth inhibitor antibiotic.

the following formulation (g/L): Yeast Nitrogen Base (6.7), as the nutrient base; ethanol (48), as the non-fermentable energy source and as an inhibitor for some of the yeasts; *p*-cumaric acid (0.1), as the phenol-like producing aroma substract; bromocresol green (~~0.222~~ 0.022), previously dissolved in NaOH, as the acid-base indicator; cycloheximide (0.01), as the inhibitor antibiotic for some of the yeast species; chloramphenicol (0.1) and/or oxytetracycline (0.1), as the bacteria inhibitor antibiotic; and agar-agar (20), as the gelling agent. The culture medium is sterilized according to the following: the agar-agar is dissolved in 70% of the total needed water, the pH is adjusted to 5.4 with a strong acid, and the resulting solution is sterilized in an autoclave at 120°C, for 20 minutes. The other components are dissolved in the remaining of the demineralized water, the pH is adjusted to 5.4 with a strong acid, and the resulting solution is sterilized by filtration through a 0.22 µm pore diameter membrane. Both of the above solutions are then mixed together when the agar-agar solution reaches 50°C. The medium is then homogenized and dispensed into Petri dishes, allowing it to solidify prior to the inoculation.

Example 2:

Use of the culture medium object of the invention for the detection of yeasts of the* Dekkera *and* Brettanomyces *genera in wines

In this example, two wines suspected of having been altered were analyzed using the culture medium of Example 1. 20 ml samples of each wine were filtered under aseptic conditions, through 0,22 µm pore diameter cellulose acetate membranes. Each membrane was placed on the surface of a Petri dish containing the medium of the invention and incubated at 25°C. After 3 days it was possible to observe colonies in one of the dishes, along with the change of the medium color from blue to yellow; when the dish was opened, the presence of a phenol-like aroma was not detected. In the other dish no colonies were detect after 3 days. After 9 days the dish where the colonies had been observed maintained the same characteristics. In the other dish it was possible to observe small cream colored colonies, a color change of the

CLAIMS

1 – A differential culture medium for the enumeration of food and beverage contaminant yeasts of the *Dekkera* and *Brettanomyces* genera, characterized in that it comprises a nutrient base, ethanol, *p*-cumaric acid, an acid-base indicator with turning points in the acid range, an inhibitor antibiotic for ~~some~~ sensitive yeasts species, and optionally a bacteria growth inhibitor and agar-agar.

2 – A culture medium according to claim 1, characterized in that the present amount of ethanol is from 32 to 96 g/L, preferably 48 g/L.

3 – A culture medium according to claim 1, characterized in that the present amount of *p*-cumaric acid is from 0.05 to 1.0 g/L, preferably 0.1 g/L.

4 – A culture medium according to claim 1, characterized in that the nutrient base is "Yeast Nitrogen Base", in amounts from 5 to 10 g/L, preferably 6.7 g/L.

5 – A culture medium according to claim 1, characterized in that the inhibitor antibiotic for some of the yeast species is cycloheximide, present in an amount from 0.004 to 0.1 g/L, preferably 0.01 g/L.

6 – A culture medium according to claim 1, characterized in that the pH indicator is bromocresol green, present in an amount of ~~about~~ 0.022 g/L.

7 – A culture medium according to claim 6, characterized in that the medium pH is adjusted with a strong acid to a value between 4.8 and 6.0, preferably 5.4.

8 – A culture medium according to claim 1, characterized in that it additionally contains a bacteria growth inhibitor, preferably chloramphenicol and/or

oxytetracycline, in amounts of ~~about~~ 0.1 g/L, to detect and identify yeasts of the *Dekkera* and *Brettanomyces* genera in food and beverage products containing mixed populations of yeasts and bacteria.

9 – A culture medium according to any one of the preceding claims, characterized in that it contains all the components except agar-agar, to detect and identify yeasts of the *Dekkera* and *Brettanomyces* genera in food and beverage products containing mixed populations of yeasts, bacteria and particularly filamentous fungi.

10 – A differential culture medium for enumeration of food and beverage contaminant yeasts of the *Dekkera* and *Brettanomyces* genera, characterized in that it has the following composition: 5 to 10 g/L, preferably 6.7 g/L, of "Yeast Nitrogen Base"; 0.004 to 0.1 g/L, preferably 0.01 g/L, of cycloheximide; 0.05 to 1.0 g/L, preferably 0.1 g/L, of *p*-cumaric acid; 0.022 g/L of bromocresol green, or another acid-base indicator with similar turning points; 32 to 96 g/L, preferably 48 g/L, of ethanol; 0.1 g/L of chloramphenicol and/or 0.1 g/L of oxytetracycline, and 20 g/L of agar-agar, the pH of the medium being adjusted between 4.8 and 6.0, preferably 5.4, with a strong acid.

11– A differential culture medium for enumeration of food and beverage contaminant yeasts of the *Dekkera* and *Brettanomyces* genera according to the preceding claims, characterized by the sterilization of all the components is done by filtration, except for the agar-agar which is sterilized in autoclave; the addition under aseptic conditions to this solution, after agar-agar cooling and before it solidifies, of all the other components of the medium, previously sterilized by filtration; and the dispensing of the medium into Petri dishes so that it solidifies.

12 – A process for the detection and/or identification of yeasts of the *Dekkera*

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/PT 00/00005

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	P. CHATONNET ET AL.: "The origin of ethylphenols in wines" JOURNAL OF THE SCIENCE OF FOOD AND AGRICULTURE., vol. 60, no. 2, 1992, pages 165-178, XP002148620 ELSEVIER APPLIED SCIENCE PUBLISHERS. BARKING., GB ISSN: 0022-5142 cited in the application page 166, column 2 -page 167, column 2 --- -/--	1-16

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 September 2000	13/10/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Griffith, G

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte Ional Application No
PCT/PT 00/00005

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 122, no. 9, 27 February 1995 (1995-02-27) Columbus, Ohio, US; abstract no. 101322, D. A. N. EDLIN ET AL.: "The biotransformation of simple phenolic compounds by Brettanomyces anomalus" page 574; column 1; XP002148621 abstract & FEMS MICROBIOL. LETT., vol. 125, no. 2-3, 1995, pages 311-316, ---	1-16
A	CHEMICAL ABSTRACTS, vol. 106, no. 3, 19 January 1987 (1987-01-19) Columbus, Ohio, US; abstract no. 15531, T. HERESZTYM: "Metabolism of volatile phenolic compounds from hydroxycinnamic acids by Brettanomyces yeast" page 331; column 1; XP002148622 abstract & ARCH. MICROBIOL., vol. 146, no. 1, 1986, pages 96-98, -----	1-16

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PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:
FERREIRA MAGNO, Fernando António
Rua das Flores, 74 - 4º andar
P-1200-195 Lisboa
PORTUGAL

Date of mailing (day/month/year)	
07 December 2000 (07.12.00)	
Applicant's or agent's file reference	**IMPORTANT NOTICE**
84 809	

International application No.	International filing date (day/month/year)	Priority date (day/month/year)
PCT/PT00/00005	31 May 2000 (31.05.00)	31 May 1999 (31.05.99)

Applicant
INSTITUTO SUPERIOR DE AGRONOMIA et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AG,AU,DZ,KP,KR,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CN,CR,CU,CZ,DE,DK,DM,EA,EE,EP,ES,FI,GB,GD,
   GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MN,MW,MX,
   NO,NZ,OA,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,ZA,ZW
   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 48.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   07 December 2000 (07.12.00) under No. WO 00/73495

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, a demand for international preliminary examination must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the national phase, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer
J. Zahra

Telephone No. (41-22) 338.83.38

Continuation of Form PCT/IB/308

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

Date of mailing (day/month/year)	IMPORTANT NOTICE
07 December 2000 (07.12.00)	
Applicant's or agent's file reference	**International application No.**
84 809	PCT/PT00/00005

The applicant is hereby notified that, at the time of establishment of this Notice, the time limit under Rule 46.1 for making amendments under Article 19 has not yet expired and the International Bureau had received neither such amendments nor a declaration that the applicant does not wish to make amendments.

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

FERREIRA MAGNO, Fernando Antnio
Rua das Flores, 74 - 4 andar
P-1200-195 Lisboa
PORTUGAL

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	23.08.2001
Applicant's or agent's file reference: 84 809	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/PT00/00005	31/05/2000	31/05/1999

Applicant
INSTITUTO SUPERIOR DE AGRONOMIA et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Danti, B Tel.+49 89 2399-8161

Form PCT/IPEA/416 (July 1992)

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PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
84 809		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/PT00/00005	31/05/2000	31/05/1999
International Patent Classification (IPC) or national classification and IPC C12Q1/04		
Applicant INSTITUTO SUPERIOR DE AGRONOMIA et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 6 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
15/12/2000	23.08.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Thumb, W Telephone No. +49 89 2399 7350

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/PT00/00005

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1,3-5,8	as originally filed			
2,6,6a,7	as received on	06/06/2001	with letter of	04/06/2001

Claims, No.:

12 (part),13-16	as originally filed			
1-11,12 (part)	as received on	06/06/2001	with letter of	04/06/2001

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   ☐ the language of publication of the international application (under Rule 48.3(b)).
   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☐ contained in the international application in written form.
   ☐ filed together with the international application in computer readable form.
   ☐ furnished subsequently to this Authority in written form.
   ☐ furnished subsequently to this Authority in computer readable form.
   ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:
   ☐ the claims, Nos.:
   ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-16
	No:	Claims	
Inventive step (IS)	Yes:	Claims	10
	No:	Claims	1-9, 11-16
Industrial applicability (IA)	Yes:	Claims	1-16
	No:	Claims	

2. Citations and explanations
   see separate sheet

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. The amendments filed with the letter dated 4.6.2001 do not appear to introduce subject-matter which extends beyond the content of the application as originally filed and therefore comply with the provisions of Article 34(2)(b) PCT.

2. Reference is made to the following document:

   D1: P. CHATONNET ET AL.: 'The origin of ethylphenols in wines' JOURNAL OF THE SCIENCE OF FOOD AND AGRICULTURE., vol. 60, no. 2, 1992, pages 165-178, cited in the application.

3. Novelty

   Claims 1-16, referring to a differential culture medium for the enumeration of yeasts of the *Dekkera* and *Brettanomyces* genus, containing, among other components, ethanol and p-coumaric acid, and the use of said medium, are novel within the meaning of Article 33(2) PCT, since said medium is not known from the state of the art known to the examining authority.

4. Inventive step

4.1 Document D1, which is considered to represent the most relevant state of the art, discloses media for culturing yeasts of the genus *Dekkera* and *Brettanomyces.*
A solid medium comprises agar (20 g/l), nutrition components, bromocresol green (0.03 g/l), cycloheximide (0.005 g/l), and 3% ethanol, the latter resulting from the addition of penicillin, gentamicin sodium sulphate and diphenyl in ethanol-solution to the medium. The pH is adjusted to 4.8 (page 167, column 1, lines 37-51).
The decarboxylation of p-coumaric acid by the yeasts is investigated in YPG liquid medium, containing 100 mg/l p-coumaric acid. The culture is continued for 3 days (page 167, column 2, lines 10-27).
The only yeasts which are capable of synthesising the 4-ethylphenol from the p-coumaric acid are *Brettanomyces/Dekkera* (page 171, column 2, lines 15-19).

4-Ethylphenol is known to possess an intense odour (page 168, column 2, lines 7-9).

4.2 The subject-matter of present claim 1 differs from the teaching of D1 in that p-coumaric acid is added to the *Dekkera/Brettanomyces*-specific medium, described in paragraph 1 of item 4.1 above.
The underlying objective problem may therefore be seen in including an additional compound in a medium suitable for detection of yeasts of the *Dekkera/ Brettanomyces* genera into a culture medium.
However, since it is already mentioned in D1 that *Dekkera* and *Brettanomyces* are the only yeasts capable of transforming p-coumaric acid into 4-ethylphenol and the characteristic odour of said compound is also well known in the art it would be obvious for the skilled person to include p-coumaric acid into a cell culture medium for specific amplification of the above-mentioned yeasts to arrive at a solution to the above stated problem.
The applicant argues that the essential technical feature of the present application is the use of ethanol as the only non-fermentable energy source in the medium. This is, however, not reflected by the broad wording of claim 1, which refers to a medium comprising (emphasis added) components including ethanol and p-coumaric acid. It is therefore not excluded that additional components may be present in the medium of claim 1 (e.g. sugars as an additional energy source). In the present wording, the only difference between the subject-matter of claim 1 and the culture medium disclosed in D1 is the inclusion of p-coumaric acid in the medium which cannot be considered as being inventive following the argumentation put forward in the preceding paragraphs.
Claim 1 therefore does not meet the requirements of Article 33(3) PCT.

4.3 Dependent claims 2-9 and 11 do not contain any features which, in combination with the features of any claim to which they refer, meet the requirements of the PCT in respect of inventive step, the reasons being as follows:
The amount of ethanol specified in claim 2 appears to be normal design option which the skilled person would choose in, accordance with circumstances, taking into consideration that the inhibitory effect of ethanol on the growth of a number of microorganisms is well known in the art.
The subject-matter of claims 3, 5, and 7 is disclosed in D1 (see item 3.1 above).

The amount of bromocresol green disclosed in claim 6 differs only marginally from the amount used in D1.
The subject-matter of claims 4 and 8 appears to be a mere selection among a number of known possibilities, not associated with a surprising technical effect, from which the skilled artisan would select without the exercise of an inventive activity (see also PCT Guidelines, IV-8.8(C1)(i)).
Claim 9 is obvious since the use of a medium with a specific composition either as a liquid or as a solid medium is trivial.
Choosing a certain order of mixing compounds of a medium and the selection of suitable sterilization steps (claim 11) is a practice well known to the person skilled in the art, and as such not suitable to confer an inventive activity.
Claims 2-9 and 11 therefore do not meet the requirements of Article 33(3) PCT.

4.4 Claims 12-16 referring to the use of said medium to detect *Dekkera/Brettanomyces* in food and beverages and for culturing said organisms is obvious for the skilled person, for example in view of the teaching of D1, referring to the detection of *Brettanomyces/Dekkera* in red wine, and wherein also the negative effects of phenolic compounds produced by said yeasts on the beverage quality are acknowledged (see page 165, abstract, lines 1-9).
Claims 12-16 can therefore also not be considered as comprising inventive subject-matter (Article 33(3) PCT).

4.5 Claim 10 differs from the teaching of document D1 in that it has a defined composition containing ethanol as the only non-fermentable energy source and p-coumaric acid.
The underlying problem may therefore be seen in providing a medium having a composition for selective growth of yeasts of the *Dekkera* and *Brettanomyces* genera.
The known state of the art does neither disclose nor render obvious that the use of a medium containing ethanol as the only non-fermentable energy source can be used to provide for differential growth of *Dekkera* and *Brettanomyces* genera compared to other yeast and bacterial species.
Hence, claim 10 meets the requirements of Article 33(3) PCT.

enumeration, as well as their later identification in food and beverages, rather difficult, this being generally accomplished through the use of very slow, work intensive, and technical skill demanding classical techniques or through molecular biology techniques, involving the use of expensive reactants, molecular probes or primers not always promptly available in the market, and of skilled operators.

It was possible to establish beyond any doubt that these yeasts are involved in the production of a serious organoleptic defect in wines - "horse sweat" - particularly in those that are aged in oak casks. (Chatonnet, *et al.* 1992, *J. Sc. Food Agric.*, 60,165-178). Since then, their detection and enumeration in wines became essential, arising the need for the development of swift methods for that effect. The field bibliography discloses suitable means for the detection and enumeration of yeasts of the *Dekkera* and *Brettanomyces* genera based on this species resistance to cycloheximide and their acidifying ability (Chatonnet, *et al.* 1992, *J. Sc. Food Agric.*, 60,165-178; Fugelsang, K. *et al.*1993. Ed. Barry H. Gump. ACS Symposium series 536, *American Chemical Society*, Washington. Cap. 7, 110-119; Alguacil, M. *et al.* 1998. *Aliment. Equipos Tecnol*, 10, 81-85). However, the disclosed media were not entirely satisfactory, since they were not selective enough to prevent the growing of fast developing species, and also were not totally differential.

Therefore, there is a real and effective need for a culture medium and method for the easy and swift identification of yeasts of the *Dekkera* and *Brettanomyces* genera, namely to provide the food and beverage industry a swift method for the isolation, differential detection and enumeration of yeasts of the *Dekkera* and *Brettanomyces* genera.

Description of the Invention

It was surprisingly found that using a culture medium containing ethanol and *p*-cumaric acid for *Dekkera* and *Brettanomyces* genera yeasts growth, these

Further, the medium according to the invention is useful for inclusion in yeast identification galleries.

Preferred Embodiments of the Invention

In a preferred embodiment, the present invention refers to a culture medium for the differential detection and enumeration of yeasts of the *Dekkera* and *Brettanomyces* genera which comprises "Yeast Nitrogen Base" as a nutrient base, ethanol as a non-fermentable energy source, *p*-cumaric acid as an aromatic compound (4-ethylphenol) promoting substrate, bromocresol green as an acid-base indicator with turning points in the acid range, an antibiotic that inhibits several species of yeasts (cycloheximide), and a bacterial growth inhibitor antibiotic.

In this embodiment of the invention, after culture medium inoculation with a sample containing yeasts of the *Dekkera* and *Brettanomyces* genera, which may be a previously isolated sample of these yeasts, or a mixed sample of yeasts and/or yeasts and bacteria, and incubation under advantageous growth conditions for these yeasts genera, after about 5 to 12 days, identification is possible by a culture medium color change, from blue to yellow, development of cream colored colonies and the characteristic phenol-like aroma.

The present invention is further illustrated by means of the following examples, which are intended only to exemplify and by no means limit the scope of the invention.

Examples

Example 1:

Preparation of a culture medium according to the invention

The culture medium, object of the present invention, can be prepared using

the following formulation (g/L): Yeast Nitrogen Base (6.7), as the nutrient base; ethanol (48), as the non-fermentable energy source and as an inhibitor for some of the yeasts; *p*-cumaric acid (0.1), as the phenol-like producing aroma substract; bromocresol green (0.222), previously dissolved in NaOH, as the acid-base indicator; cycloheximide (0.01), as the inhibitor antibiotic for some of the yeast species; chloramphenicol (0.1) and/or oxytetracycline (0.1), as the bacteria inhibitor antibiotic; and agar-agar (20), as the gelling agent. The culture medium is sterilized according to the following: the agar-agar is dissolved in 70% of the total needed water, the pH is adjusted to 5.4 with a strong acid, and the resulting solution is sterilized in an autoclave at 120°C, for 20 minutes. The other components are dissolved in the remaining of the demineralized water, the pH is adjusted to 5.4 with a strong acid, and the resulting solution is sterilized by filtration through a 0.22 µm pore diameter membrane. Both of the above solutions are then mixed together when the agar-agar solution reaches 50°C. The medium is then homogenized and dispensed into Petri dishes, allowing it to solidify prior to the inoculation.

Example 2:

Use of the culture medium object of the invention for the detection of yeasts of the* Dekkera *and* Brettanomyces *genera in wines

In this example, two wines suspected of having been altered were analyzed using the culture medium of Example 1. 20 ml samples of each wine were filtered under aseptic conditions, through 0,22 µm pore diameter cellulose acetate membranes. Each membrane was placed on the surface of a Petri dish containing the medium of the invention and incubated at 25°C. After 3 days it was possible to observe colonies in one of the dishes, along with the change of the medium color from blue to yellow; when the dish was opened, the presence of a phenol-like aroma was not detected. In the other dish no colonies were detect after 3 days. After 9 days the dish where the colonies had been observed maintained the same characteristics. In the other dish it was possible to observe small cream colored colonies, a color change of the

CLAIMS

1 – A differential culture medium for the enumeration of food and beverage contaminant yeasts of the *Dekkera* and *Brettanomyces* genera, characterized in that it comprises a nutrient base, ethanol, *p*-cumaric acid, an acid-base indicator with turning points in the acid range, an inhibitor antibiotic for some yeasts species, and optionally a bacteria growth inhibitor and agar-agar.

2 – A culture medium according to claim 1, characterized in that the present amount of ethanol is from 32 to 96 g/L, preferably 48 g/L.

3 – A culture medium according to claim 1, characterized in that the present amount of *p*-cumaric acid is from 0.05 to 1.0 g/L, preferably 0.1 g/L.

4 – A culture medium according to claim 1, characterized in that the nutrient base is "Yeast Nitrogen Base", in amounts from 5 to 10 g/L, preferably 6.7 g/L.

5 – A culture medium according to claim 1, characterized in that the inhibitor antibiotic for some of the yeast species is cycloheximide, present in an amount from 0.004 to 0.1 g/L, preferably 0.01 g/L.

6 – A culture medium according to claim 1, characterized in that the pH indicator is bromocresol green, present in an amount of about 0.022 g/L.

7 – A culture medium according to claim 6, characterized in that the medium pH is adjusted with a strong acid to a value between 4.8 and 6.0, preferably 5.4.

8 – A culture medium according to claim 1, characterized in that it additionally contains a bacteria growth inhibitor, preferably chloramphenicol and/or

oxytetracycline, in amounts of about 0.1 g/L, to detect and identify yeasts of the *Dekkera* and *Brettanomyces* genera in food and beverage products containing mixed populations of yeasts and bacteria.

9 – A culture medium according to any one of the preceding claims, characterized in that it contains all the components except agar-agar, to detect and identify yeasts of the *Dekkera* and *Brettanomyces* genera in food and beverage products containing mixed populations of yeasts, bacteria and particularly filamentous fungi.

10 – A differential culture medium for enumeration of food and beverage contaminant yeasts of the *Dekkera* and *Brettanomyces* genera, characterized in that it has the following composition: 5 to 10 g/L, preferably 6.7 g/L, of "Yeast Nitrogen Base"; 0.004 to 0.1 g/L, preferably 0.01 g/L, of cycloheximide; 0.05 to 1.0 g/L, preferably 0.1 g/L, of *p*-cumaric acid; 0.022 g/L of bromocresol green, or another acid-base indicator with similar turning points; 32 to 96 g/L, preferably 48 g/L, of ethanol; 0.1 g/L of chloramphenicol and/or 0.1 g/L of oxytetracycline, and 20 g/L of agar-agar, the pH of the medium being adjusted between 4.8 and 6.0, preferably 5.4, with a strong acid.

11– A differential culture medium for enumeration of food and beverage contaminant yeasts of the *Dekkera* and *Brettanomyces* genera according to the preceding claims, characterized by the sterilization of all the components is done by filtration, except for the agar-agar which is sterilized in autoclave; the addition under aseptic conditions to this solution, after agar-agar cooling and before it solidifies, of all the other components of the medium, previously sterilized by filtration; and the dispensing of the medium into Petri dishes so that it solidifies.

12 – A process for the detection and/or identification of yeasts of the *Dekkera*

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/PT 00/00005

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	P. CHATONNET ET AL.: "The origin of ethylphenols in wines" JOURNAL OF THE SCIENCE OF FOOD AND AGRICULTURE., vol. 60, no. 2, 1992, pages 165-178, XP002148620 ELSEVIER APPLIED SCIENCE PUBLISHERS. BARKING., GB ISSN: 0022-5142 cited in the application page 166, column 2 -page 167, column 2 -/--	1-16

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 September 2000	13/10/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Griffith, G

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 122, no. 9, 27 February 1995 (1995-02-27) Columbus, Ohio, US; abstract no. 101322, D. A. N. EDLIN ET AL.: "The biotransformation of simple phenolic compounds by Brettanomyces anomalus" page 574; column 1; XP002148621 abstract & FEMS MICROBIOL. LETT., vol. 125, no. 2-3, 1995, pages 311-316, ---	1-16
A	CHEMICAL ABSTRACTS, vol. 106, no. 3, 19 January 1987 (1987-01-19) Columbus, Ohio, US; abstract no. 15531, T. HERESZTYM: "Metabolism of volatile phenolic compounds from hydroxycinnamic acids by Brettanomyces yeast" page 331; column 1; XP002148622 abstract & ARCH. MICROBIOL., vol. 146, no. 1, 1986, pages 96-98, -----	1-16